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HPLC retention-time-shift determination of nitrogen isotope ratios in enriched water

STABLE ISOTOPES are used as tracers to follow the fate of elements in organisms and/or ecosystems. Stable isotope composition is usually measured by mass detection techniques (mass or emission spectrometry) that require the elements to be converted into gaseous forms before measurement.[1] Determination of nitrogen regeneration (ammonium production) rates in natural waters by the isotope dilution technique requires measnrement of ammonium concentrations and isotope ratios of ammonium-N in bottles at intervals after $^{15}NH_4^+$ is added.[2] Large samples of water (often 100–500 mL) are required to isolate sufficient quantities of N for analysis because the concentration of dissolved ammonium in natural or experimental waters is usually low relative to working ranges of mass detection techniques. Contamination of the samples with atmospheric ammonium or with nitrogen from reagents or other sources must be avoided for accurate isotope ratio measurements.[1]

An alternative technique to measure nitrogen isotope ratios of ammonium in enriched water for isotope dilution experiments is direct-injection cation-exchange HPLC with post-column derivatization and measurement of retention time shifts (RTSs).[3] With this approach, small samples of water from isotope dilution experiments can be simply filtered and analyzed directly for ammonium concentrations and isotope ratios.[4]

Quantification of isotope ratios from ammonium RTSs

Cation-exchange fractionation of the two isotopic forms of ammonium is possible because the heavy ^{15}N form of ammonium elutes at a slightly lower rate than the ^{14}N form. This elution order occurs because a slightly larger fraction of ^{15}N than ^{14}N occurs as cationic NH_4^+ in the equilibrium reaction between ammonium and ammonia at pHs near the pK (approximately pH = 9). Chromatographic columns are not efficient enough to resolve the two forms of nitrogen into separate peaks, but isotope ratios can be determined by quantifying RTSs for ammonium caused by the presence of $^{15}NH_4^+$. This HPLC approach is unique in that chromatographic peak retention time is used to quantitatively measure the composition of peak components rather than to merely qualitatively identify compounds. If two compounds have slightly different retention times but have otherwise identical chromatographic behaviors and detector responses, the RTS occurs in a predictable manner and can be quantified by determining the sample peak position, relative to that of an internal standard injected at a precise time interval before the sample.[3] These conditions are met for isotopically caused RTS in the cation-exchange fractionation of ammonium isotopes at pHs near the pK for ammonium (*Figure 1*). If retention times are evaluated using the traditional peak-maximum method, the resulting calibration curve of RTS versus fractional component is sigmoid-shaped, making the response insensitive at low and high isotope ratios.[3] These difficulties can be overcome by calculating the RTS based on the median area of the unresolved band.[5] In this case, the retention time is defined as the time required to reach the center of mass, or centroid, of the peak. Graphically, it is the time required to reach a vertical line that divides the peak into two equal areas. For two unresolved components, this method accurately measures the RTS caused by effective weighting of each component within the unresolved band and results in a linear calibration curve of RTS versus isotope enrichment.[5] With this approach, both ammonium concentration, based on peak area, and N-isotopic composition of the ammonium, based on RTS, can be obtained from the same chromatograms on ^{15}N-enriched samples from isotope addition experiments.

HPLC system description

The HPLC system, with a post-column o-phthalaldehyde 2-mercaptoethanol (OPA) reaction system (*Figure*

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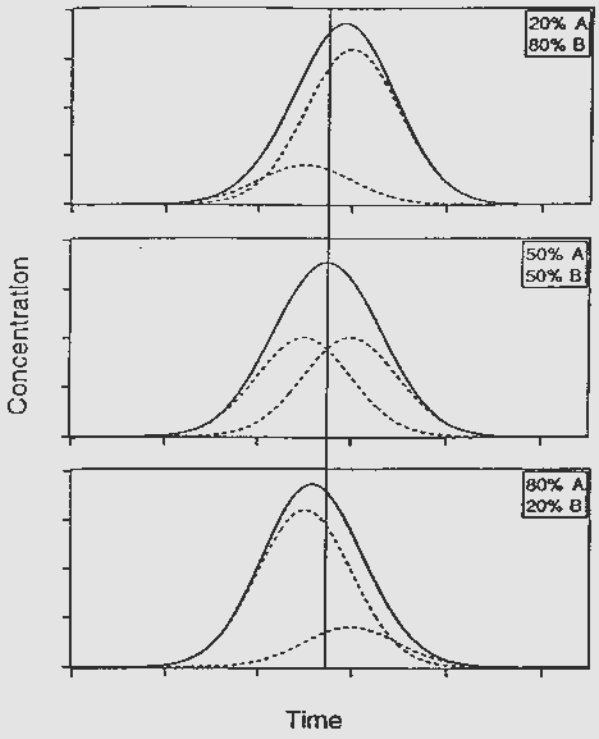

Figure 1 *Schematic diagram illustrating the contribution of two chromatographic components with slightly different retention times (e.g., $^{14}NH_4^+$ and $^{15}NH_4^+$) to the total response of the unresolved peak.*

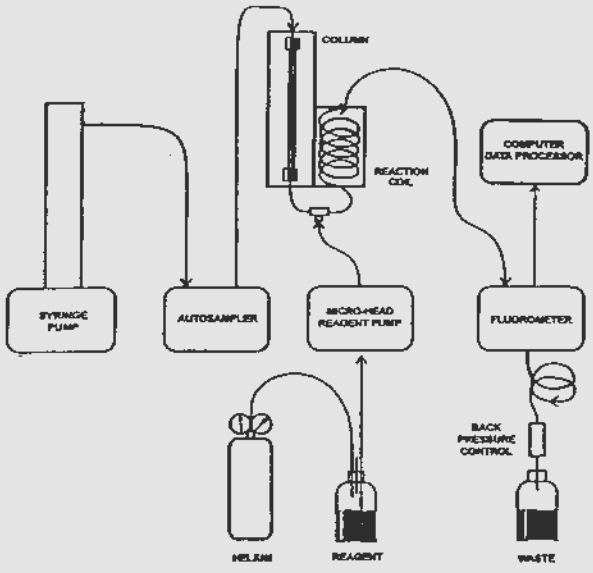

Figure 2 *Schematic diagram of HPLC apparatus used to measure both concentration and isotope ratios for ammonium dissolved in water that was enriched with $^{15}NH_4^+$ (or ^{15}N-labeled amino acids) for isotope addition experiments.*

2), is assembled from components that provide stable and precise flow conditions for both mobile-phase buffer and reagent over a period of approximately 21 hr of continuous operation. A model 260D syringe pump (ISCO, Lincoln, NE) (capacity 266 mL), operated under constant pressure (2600 psi), delivers mobile-phase buffer (a boric acid buffer adjusted to pH 9.41) at a flow rate of approximately 0.17 mL/min^{-1}. Sample filtrates are injected with a model 728 autosampler (Alcott, Norcross, GA) equipped with a model EC6W fast electronically activated injection valve (Valco, Houston, TX) with a 50-µL sample loop. The HPLC column is a strong cation-exchange resin (5-µm beads of the sodium form of sulfonic acid cation exchanger with 12% cross-linked polystyrene/divinylbenzene polymeric matrix, St. John Associates, Beltsville, MD) contained in a 30 cm × 4 mm i.d. stainless steel column. The column is maintained at 85 °C with a standard CROCO-CIL HPLC column heater (Anspec, Ann Arbor, MI). The post-column OPA reagent is delivered at 0.1 mL/min^{-1} through a T-fitting to the column outflow with a model 760 HPLC pump (Alcott) equipped with a microbore head. A slow flow of helium is maintained above the reagent solution to prevent gas bubbles from affecting RTS. The post-column mixture is passed sequentially through a heated (40 °C) 1-m PTFE microbore reaction coil, a model 121 fluorometric detector (Gilson, Middleton, WI), and a model U446 100-psi back-pressure regulator (Upchurch Scientific, Oak Harbor, WA). Chromatographic data are collected on a PC equipped with an Array Basic Grams/386 data system (Galactic Industries Corp., Salem, NH). After each chromatographic run, the median-area (centroid) retention time for each peak is calculated with a post-peak-collection analysis program (COL_RT.ABP, Galactic).[5]

The autosampler is programmed for sequential injection of internal standards and samples. The internal ammonium standard (IAS), prepared in mobile-phase buffer, is injected 7.0 min before each sample or calibration-curve standard so that RTS can be calculated by subtracting the retention time of the IAS from that of the sample after compensating for the difference in injection times. Sufficient time (typically 34 min) is programmed after sample injection to allow the sample ammonium peak to elute before the next IAS is injected. Before an analytical run, odd-numbered injection vials are loaded with IAS, and even-numbered vials are loaded with sample filtrates or calibration-curve standards (prepared in water with a salinity matrix matching that of the samples). Each sample filtrate or calibration-curve standard is analyzed sequentially three times so that RTS results can be averaged for improved precision and evaluated for quality assurance. Calibration standards containing three different atom percent enrichments of $^{15}NH_4^+$ (*Figure 3*) are typically run with each group of samples so that a separate isotope ratio calibration can be done for each analytical

run. Ammonium concentrations are calculated by comparing sample ammonium peak areas to IAS peak areas after slight corrections are made for matrix effects (e.g., salinity) based on the calibration-curve standards. Peak areas rather than peak heights are used to calculate ammonium concentrations for samples because the exact peak heights vary with isotope ratios (Figure 3).

Selected application

Zebra mussels (*Dreissena polymorpha*), recent invaders of the Great Lakes, affect lower-food-web/nutrient dynamics in shallow regions by filtering phytoplankton, protozoa, and other particles from the water and excreting nutrients.[6] Determining the effects of zebra mussel on the mechanisms and rates of nutrient cycling in coastal regions such as Saginaw Bay is important because nutrient-supply rates and ratios (e.g., N:P or NH_4^+:NO_3^-) can be important factors affecting the abundance and composition of phytoplankton and other food web organisms in lakes and coastal ecosystems.[7] Application of the method to this problem is demonstrated by observing changes in ammonium concentrations and isotope ratios in Saginaw Bay, Lake Huron, water after additions of 4 μM $^{15}NH_4^+$ under natural-light and dark conditions and in the absence and presence (15 individuals per 4 L water) of zebra mussels (*Figure 4*). The experiment was conducted in July 1994 on a water sample from a eutrophic region of the bay where the water temperature was 22 °C, and the blue-green alga, *Microcystis aeruginoso*, was dominant and abundant.

Isotope ratios decreased dramatically in all treatments, indicating that substantial ammonium remineralization was occurring in all treatment bottles. Note that, in isotope-dilution experiments, both forms of ammonium are assumed to be taken up by organisms (phytoplankton and/or bacteria) in direct proportion to their relative abundances, whereas regenerated ammonium is assumed to be produced from natural organic material that contains natural-abundance nitrogen (i.e., 99.63% ^{14}N).[2] Ammonium-concentration patterns varied among the different treatments. Net ammonium concentrations decreased in the light bottle without zebra mussels, likely due to uptake of ammonium by *Microcystis* and other phytoplankton, and increased in the dark treatment with zebra mussels, probably as a result of decreased phytoplankton uptake in the dark and zebra mussel excretion (Figure 4). Net changes in the other two treatments were less pronounced because uptake and regeneration rates were more nearly balanced. The observation that the atom percent enrichment of $^{15}NH_4^+$ decreased at a faster rate in the light treatment with zebra mussels than in the dark one without zebra mussels,

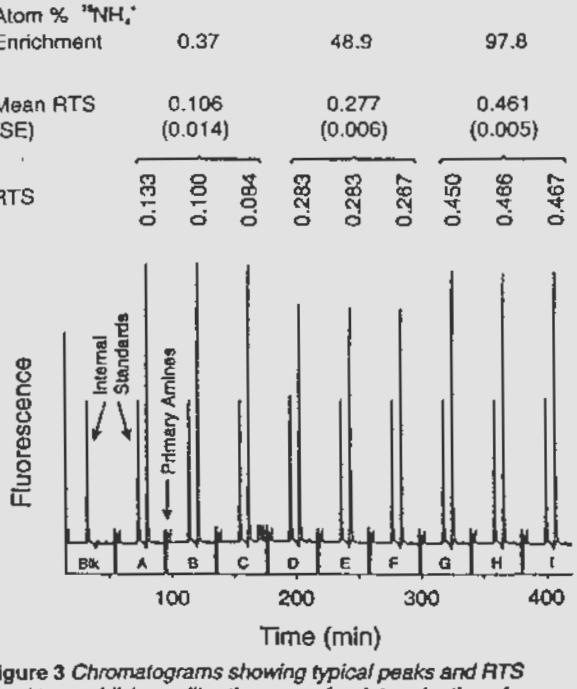

Figure 3 *Chromatograms showing typical peaks and RTS used to establish a calibration curve for determination of $^{15}NH_4^+$:$^{14}NH_4^+$ ratios. Internal standards = peaks from an internal standard of 4 µM natural-abundance NH_4^+ prepared in mobile-phase buffer. A, B, and C; D, E, and F; and G, H, and I = peaks from lake water containing 8 µM of added mixtures of NH_4^+ containing 0.37, 48.9, and 97.8 atom percent enrichments of $^{15}NH_4^+$, respectively. RTS = retention time shift (min). SE = standard error of the mean. The observed relationship between mean RTS and isotope ratios for this experiment is RTS = 0.1027 + 0.3644 (atom percent $^{15}NH_4^+$/100); correlation coefficient = 0.9998 (N = 3). The chromatographic data shown above were taken from the analytical run that quantified the isotope ratios for the dark with zebra mussel treatment shown in Figure 4.*

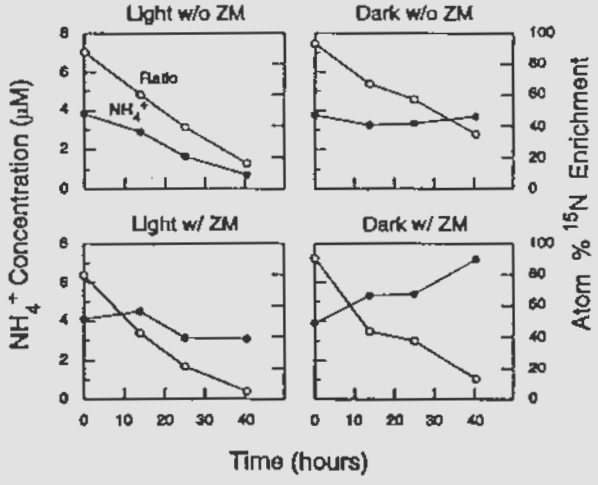

Figure 4 *Time-course patterns for ammonium concentrations and isotope ratios for light and dark Saginaw Bay isotope-dilution experiments that were conducted in the absence and presence of zebra mussels (15 individuals per 4 L water) in July 1994 when high levels of the blue-green alga, Microcystis aeruginosa, were present.*

while net ammonium-concentration changes were minimal in both of these treatments, indicates that nitrogen recycling rates were more rapid in the former treatment than in the latter one. Ammonium regeneration rates calculated over the first time interval by the Blackburn model[2] were 0.06 and 0.08 $\mu M/hr^{-1}$ for the treatments without zebra mussels as compared to 0.15 and 0.23 $\mu M/hr^{-1}$ for the two zebra mussel treatments. Thus, recycling rates for nitrogen were appreciable even though net concentration changes in ammonium over time were sometimes small.

Conclusions

Direct measurement of both ammonium concentrations and the atom percent ^{15}N enrichment of the ammonium by cation-exchange HPLC simplifies the conducting of isotope-addition experiments in natural waters. The use of RTS to quantify isotope ratios of ^{15}N:^{14}N for dissolved ammonium makes feasible the direct measurement of isotope ratios in small volumes of water with minimal handling of the samples before analysis. Sample filtrates from shipboard or other isotope-addition experiments can be frozen and stored in small containers until analysis by direct-injection HPLC. This approach minimizes exposure of the samples to the atmosphere and to reagents that could otherwise contaminate them with ammonium or other sources of nitrogen and adversely affect isotope-ratio results. Equipment is inexpensive relative to emission or mass spectrometry. Standards preparation, replenishment of buffer and reagent, loading of the autosampler for triplicate runs of three calibration standards and six samples, and recovery of data from the previous run generally require less than 2 hr of analyst time per day.

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